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(54) Title of Invention Fixed microorganisms and their production method

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Detailed Description

1. Title of Invention Fixed organisms and their production

2. Scope of Claims

1. Fixed microorganisms fixed on the cell wall surface of a ceramic honeycomb structured body having multiple parallel holes perforated, wherein a mixture of a polysaccharide and microorganisms containing enzymes is fixed such that the microorganisms containing enzymes are included in polysaccharides.

2. The fixed microorganisms of Claim 1 wherein the polysaccharide is one of agar, copper carrageenan and arginate.

3. Method for producing fixed microorganisms wherein a gel-like solution of a mixture of a polysaccharide and microorganisms containing enzymes is adhered to the

cell wall surface of a ceramic honeycomb structured body having multiple parallel holes perforated, the gel-like mixture is fixed by having this structured body come in contact with a solidifying solution such that the microorganisms containing enzymes are fixed on the cell wall surface of the ceramic honeycomb structure in a form of the microorganisms included in the polysaccharide.

4. The method for producing fixed microorganisms of Claim 3 wherein the surface crudeness of the cell wall surface of the ceramic honeycomb structured body is 10 μm~100 μm.

5. The method for producing fixed microorganisms of Claim 4 wherein the surface crudeness is controlled by adhering ceramic particles of sizes 200 μm~1,000 μm onto the cell surface.

6. The method for producing fixed microorganisms of Claim 4 wherein a gel-like mixture is adhere-fixed on the cell wall surface in a thickness of 100 μm~1,000 μm.

7. The method for producing fixed microorganisms of Claim 3 wherein the cell opening length of the ceramic honeycomb structured body is 2 mm~10 mm.

3. Detailed description of invention

The present invention relates to fixed microorganisms and their production method wherein microorganisms containing enzymes used in biochemical reactions are fixed on a catalyst of a ceramic honeycomb structured body.

Recently, biochemical reactions using microorganisms have been rapidly developed and have had wide applications in the fields of organic synthesis, food industry, analytical chemistry etc. As methods for fixing microorganisms containing enzymes used in these biochemical reactions, the following methods are known: a covalent bonding method that covalent-bonds microorganisms containing enzymes with various kinds of bead or pellet form water-insoluble carriers; a bridging method that bridges microorganisms with carriers using bridging reagents that have two or more than two functional groups such as glutaraldehyde, bisdiazobenzidine etc.; so-called inclusion methods that enclose microorganisms inside a polymer gel frame of polyacrylamide, starch, carrageenan etc. or cover microorganisms with semitransparent polymer film.

However, the covalent bonding or bridging methods have a shortcoming of changing the properties of the microorganisms and decreasing their activities significantly during fixing while the inclusion methods have a problem of decreasing activities of the microorganisms during inclusion operations. The forms of adhere-fixing microorganisms to produce fixed microorganisms include in addition to the above mentioned bead and pellet form, the cyclo form, the film form etc. However, with such forms, when a high viscosity starch solution of a high concentration is passed through a substrate in the starch saccharification reaction, which is a well-known enzyme reaction, for example, problems of increasing pressure loss, obstruction of the pathway due to solid substances etc. occur. In addition, since gas is generated during the reaction in methane fermentation using methane-generating microorganisms or alcohol fermentation using yeast etc., a filling bed can easily be obstructed due to gas accumulating in a part of the filling bed, and a shearing force is added to gel particles by the gas generated, leading to impairing the particles.

A goal of the present invention is to provide for fixed microorganisms having a high adhesion strength with a minimal decrease in their activities and their production method. In addition, another goal of the present invention is to provide for fixed

microorganisms having large contact surfaces with the reactants and their production method by solving the problems of the pressure loss or the obstruction as well as facilitating dispersion of the gas occurring.

The present invention is fixed microorganisms produced by adhere-fixing a mixture of a polysaccharide and microorganisms on a cell wall surface of a ceramic honeycomb structured body having multiple parallel holes perforated such that the microorganisms containing enzymes are included in the polysaccharide.

In addition, the present invention is a method for producing fixed microorganisms that are adhere-fixed on a cell wall surface such that the microorganisms containing enzymes are included in a polysaccharide, by preparing a mixture of the polysaccharide and microorganisms containing enzymes as a gel-like solution, adhering this gel-like mixture on a cell wall surface of a ceramic honeycomb structured body having multiple parallel holes perforated, and solidifying the gel-like mixture by having the above-said structured body come in contact with a solidifying solution.

The polysaccharides used in the present invention are agar, copper carrageenan, arginate etc. In addition, the microorganisms and the enzymes contained in these microorganisms usable in the present invention are not specially limited. For example, the microorganisms used include bacteria, radioactive bacteria, molds, yeasts, etc., and the enzymes include glucoamylase, aminoamidase, glucose isomerase, β-galactosidase, cellulase, invertase, asparaginase, aspartase, catalase, protease, lipase, lysine decarboxylase, hexokinase, tryptophan synthase, glycerol dehydrogenase etc.

The ceramic honeycomb structured body used in the present invention is constructed from ceramic materials such as aluminum, murite, cordierite etc. This is because the ceramic honeycomb structured body has advantages of having the surface state optimal for fixing microorganisms containing enzymes, guaranteeing a large contact surface and incurring minimal pressure loss. The cell opening length of this ceramic honeycomb structured body is 2 mm~10 mm, or 3 mm~7 mm preferably. Adhesion of the gel-like mixture is not easy or uniform, and the pathway is obstructed, increasing the pressure loss suddenly, if the cell opening length is less than 2 mm, whereas the mechanical strength decreases and the volume of the honeycomb structured body necessary for the reaction becomes too large if the cell opening length is greater than 10 mm. Since the cell wall surface of this structured body increases the strength of the gel-like mixture, the surface crudeness is adjusted to be 10 μm~100 μm, or 30 μm~70 μm more preferably. The gel-like mixture can easily separate if the surface crudeness is less than 10 μm, whereas the mechanical strength decreases if the surface crudeness is greater than 100 μm. In order to obtain a desirable surface crudeness, in an example, ceramic particle powder of sizes of 200 μm~1,000 μm is adhered to a cell wall surface of a ceramic honeycomb structured body by extrusion molding, and then fixed on it by burning. It is desirable to perforate a multiple number of holes on the ceramic honeycomb structured body in parallel in order to avoid the problem of obstruction and to increase the contact surface area. The holes are 50~500 in number perforated on the ceramic honeycomb structured body of a 50 mm angle.

The mixture of a polysaccharide and microorganisms containing enzymes is made into a gel-like solution. A reason for this is to enclose microorganisms inside the gel frame. Although the gel-like solution differs depending on the kinds and properties of the substances mixed, the solution temperature is maintained at 30 degrees Celsius ~70

degrees Celsius. Reasons for including the microorganisms in a polysaccharide are that the polysaccharides serve as a source of nutrition for the microorganisms containing enzymes and that the decrease in the enzyme activity is minimized.

The gel-like solution is adhered on the cell wall surface of the ceramic honeycomb structured body. In this case, immersion is optimal for adhesion, although spraying or infusion etc. may also be used. Although the time of immersion of the ceramic honeycomb structured body in the gel-like solution differs depending on the gel-like solution used, the usual time of immersion is about 30 seconds to 10 minutes. The gel that has adhered on the ceramic honeycomb structured body is taken out of the gel-like solution and excess gel is blown away by compressed air etc., to obtain an arbitrary film thickness. The film thickness of the gel-like substance adhered on this cell wall surface should be 100 μm~1,000 μm in order to obtain an amount of enzymes necessary to perform subsequent biochemical reactions. Thus, this film thickness is arbitrarily established from the amount of enzymes needed for the reactions.

In order to solidify the gel-like mixture that has been adhered on the cell wall surface of the ceramic honeycomb structured body, this structured body is immersed in cold water, KCl solution, $CaCl_2$ solution, $AlCl_2$ solution, glutaraldehyde solution, hexamethylene diamine solution, $Al_2(SO_4)_8$ solution etc. so that the structured body comes in contact with the solidifying solution. The microorganisms are thus adhered-solidified in a form of being included inside a polysaccharide on the cell wall surface of the ceramic honeycomb structure. The time of contact with the solidifying solution should be 30 seconds to 10 minutes. This time can be selected arbitrarily by the concentration of the polysaccharide and the cell opening length of the ceramic honeycomb structured body etc.

The fixed microorganisms obtained thus have a high mechanical strength and thus can be used stably and continuously for a long time in reaction systems using high viscosity solutions or reaction systems that entail gas generation, since they are adhered in a film form in an almost constant thickness on a cell wall surface in a form of being included inside a polysaccharide.

The present invention is explained with examples below.

Example 1

Agar, copper carrageenan and sodium arginate shown in Table 1 as polysaccharides are dissolved in water such that the concentration of each of the polysaccharides becomes 3 weight %. As microorganisms containing enzymes, Escherichia coli E 106, which contain hydrolytic enzyme β-galactosidase, were dissolved in water such that the bacterial suspension solution concentration became 5 weight %. 10 m*l* of this bacterial suspension solution was taken and added to a water solution of polysaccharides 8 times each in amount, to prepare mixtures of polysaccharides and microorganisms containing enzymes.

In the case of agar used as polysaccharide, the mixture was maintained at 55 degrees Celsius and made into a gel-like solution, and an aluminum ceramic honeycomb structured body was immersed in this gel-like solution for 60 seconds to adhere the gel-like mixture onto the cell wall surface of this structured body. This structured body had the surface crudeness and the cell opening length shown in Table 1. Excess gel-like mixture that adhered on the cell wall surface was blown away by compressed air to adjust the adhered film thickness to the thickness shown in Table 1. Next, the gel-like mixture

was adhere-fixed on the cell wall surface of the ceramic honeycomb structured body by immersing it in a solidifying solution of water maintained at 3 degrees Celsius for 2 minutes.

In addition, in the case of copper carrageenan used as polysaccharide, the mixture was maintained at 37 degrees Celsius and made into a gel-like solution as in the case of agar, and the ceramic honeycomb structured body is immersed in this gel-like solution such that the gel-like mixture adhered onto the cell wall surface of this structured body. Next, after adjusting the adhesion film thickness using compressed air, the gel-like mixture was adhere-fixed on the cell wall surface by immersing it in a solidifying solution of potassium chloride solution of 2 weight %.

In addition, in the case of sodium arginate used as polysaccharide, operations similar to those performed for the above-said case of copper carrageenan were performed and the gel-like mixture was adhere-fixed on the cell wall surface using calcium chloride solution of 0.4 mole as solidifying solution.

The enzyme activities were measured in the fixed microorganisms N.1~No.19 of the present invention that had been adhered-fixed on the cell wall surfaces of the ceramic honeycomb structured bodies, with the microorganisms included in the polysaccharides. In addition, for comparison, polymers other than the polysaccharides such as polyacrylamide, collagen, polyvinyl alcohol were used as fixing materials and the enzyme activities were measured in these reference examples No.20~No.22. In measuring the enzyme activities, milk sugar-like substance, 2-nitrophenyl-β-D-galactopyranoside, was used as substrate. Since this substance decomposed into 0-nitrophenol and galactose by β-galactosidase, the enzyme activity was determined by measuring the amount of 0-nitrophenol at 420 nm of absorbance.

Table 1 shows the results.

Table 1

第 1 表

	実験№	多糖類物質	付着させたセラミック粒子粉末の粒径(μm)	ハニカム構造体のセル壁面の表面粗さ(μm)	セル開口長さ(mm)	混合物の付着膜厚 (μm)	単位固体量当りの酵素活性 × 10^4(U/g固体)
本発明	1	寒天	–	10	2	100	3.38
	2	〃	–	15	5	150	3.28
	3	〃	–	20	5	150	3.33
	4	〃	200	40	5	300	4.55
	5	〃	400	50	2	160	4.40
	6	〃	500	50	5	350	4.02
	7	〃	800	60	7	300	4.47
	8	〃	500	80	10	1000	4.55
	9	カッパーカラギーナン	–	10	2	350	3.40
	10	〃	–	25	2	250	3.55
	11	〃	500	50	7	500	3.88
	12	〃	1000	70	1	100	3.22
	13	〃	1300	70	5	800	3.55
	14	〃	1000	70	7	750	5.20
	15	〃	1000	100	10	1000	4.86
	16	アルギン酸ナトリウム	–	15	5	200	3.17
	17	〃	–	25	5	200	3.20
	18	〃	600	50	5	200	3.72
	19	〃	1000	70	5	300	3.85
参考例	20	ポリアクリルアミド	300	30	5	250	2.17
	21	コラーゲン	1000	70	7	300	1.85
	22	ポリビニルアルコール	500	50	5	250	1.45

Row 1: Experiment No.		size (μm) of ceramic particle powder adhered	surface crudeness (μm) of cell wall surface of honeycomb structured body	cell opening length (mm)	mixture adhesion film thickness (μm)	enzyme activity x 10^3 per unit amount of microorganisms (U/g microorganisms)
Row 2: 1	agar	-	10	2	100	3.23
Row 3: 2	agar	-	15	5	150	3.28
Row 4: 3	agar	-	20	5	150	3.33
Row 5: 4	agar	200	30	5	300	4.55
Row 6: 5	agar	500	50	2	150	4.40
Row 7: 6	agar	500	50	5	350	4.92
Row 8: 7	agar	500	50	7	800	4.47
Row 9: 8	agar	500	50	10	1000	4.55
Row 10: 9	copper carrageenan	-	10	2	350	3.40
Row 11: 10	copper carrageenan	-	25	2	250	3.55
Row 12: 11	copper carrageenan	500	50	7	500	5.88
Row 13: 12	copper carrageenan	1000	70	1	100	3.22
Row 14: 13	copper carrageenan	1000	70	5	300	6.55
Row 15: 14	copper carrageenan	1000	70	7	750	6.20
Row 16: 15	copper carrageenan	1000	100	10	1000	4.85
Row 17: 16	sodium arginate	-	15	5	200	3.17
Row 18: 17	sodium arginate	-	25	5	200	3.20
Row 19: 18	sodium arginate	500	50	5	200	3.72
Row 20: 19	sodium arginate	1000	70	5	300	3.65
Row 21: 20	polyacrylamide	200	30	5	250	2.17
Row 22: 21	collagen	1000	70	7	300	1.85
Row 23: 22	polyvinyl alcohol	500	50	5	250	1.45

First column: present invention
reference examples

A cubic shape of 50 mm was used for the ceramic honeycomb structured body and a stirred type reactor was used for the reactions. Since the adhesion strength is improved by increasing the surface crudeness of the ceramic honeycomb structured body, aluminum ceramic particle powder of sizes shown in Table 1 was fixed by adhere-burning it on the cell wall surface of the surface-smooth ceramic honeycomb structured body obtained by extrusion molding. In addition, 1 U (unit) of the enzyme activity per unit amount of bacteria is the unit of activity that changes 1 micromole of the substrate for one minute.

It is recognized from Table 1 that the fixed microorganisms of the present invention show superior activities per unit amount of bacteria as compared with the reference examples.

Example 2

In this example, experiments were performed to compare the characteristics of the microorganisms fixed in a honeycomb form in accordance with the present invention and those of the conventionally fixed microorganisms in a form of beads.

Radioactive streptomyces containing glucose isomerase and pheochromogenase as enzymes were dissolved in water to prepare a bacterial suspension solution of a

concentration of 5 weight %. 10 m*l* of this suspension solution and 70 m*l* of copper carrageenan aqueous solution of a concentration of 3.5 weight % were mixed. This mixture was dropped into a calcium chloride aqueous solution of a concentration of 2 weight % to prepare a well-known fixed microorganisms fixed in the form of beads of a mean diameter of 0.4 cm.

Next, the same mixture was made into a gel-like solution by maintaining it at a temperature of 37 degrees Celsius as in Example 1, after which an aluminum ceramic honeycomb structured body was immersed in this gel-like solution for 60 minutes to adhere the gel-like mixture on the cell wall surface in an adhesion thickness of 300 μm. The ceramic honeycomb structured body used had the cell opening length of 5 mm and the cell wall surface crudeness of 50 μm and the size of a 50 mm angle. Subsequently, the gel-like mixture was fixed by immersing it in a calcium chloride aqueous solution of a concentration of 2 weight % for 3 minutes to produce the fixed microorganisms of the present invention.

The fixed microorganisms obtained thus were filled in a tube-shaped reactor of a length of 200 mm at a 50 mm angle and the enzyme activity of glucose isomerase and the pressure loss of the filling bed were measured. In measuring the enzyme activity, fructose produced was determined quantitatively by high speed liquid chromatography. In addition, the flow rate of glucose of the substrate was 1 m*l*/min.

Table 2 shows the results.

表 2 表

固定化微生物 製品	単位菌体当り 酵素活性×10³(U/g-菌体)	充填床圧力損失 (mmAg)
従来品	0.96	78
本発明品	1.31	11

Table 2

Row 1: fixed microorganisms standard product enzyme activity x 10^3 per unit amount of bacteria (U/g-bacteria) filling bed pressure loss (mmAg)
Row 2: conventional product
Row 3: product of present invention

As is clear from the results of Table 2, it has been found that the fixed microorganisms of the present invention are superior in enzyme activities showing minimal pressure losses as compared with the conventional fixed microorganisms.

Example 3

A 30 % yeast Saccharomyces cerevisiae suspension solution and 3 weight % sodium arginate aqueous solution were made into a gel-like solution by mixing them at a temperature of 40 degrees Celsius. A murite ceramic honeycomb structured body of a size of a 50 mm angle, having the cell opening length of 5 mm and the cell wall surface crudeness of 50 μm, was immersed in this solution. Next, the adhesion thickness was made to be 200 μm by applying compressed air to blow away the gel that has adhered, and the gel-like mixture is fixed by immersing it inside aluminum sulfate 3 weight %

solution for 2 minutes to obtain honeycomb-form fixed microorganisms of the present invention.

Next, the gel-like mixture was dropped into aluminum sulfate 3 weight % solution as described above to obtain microorganisms fixed in the form of beads of a mean diameter of 0.3 cm (conventional product).

The microorganisms obtained thus are filled in a tube-shaped reactor of the size same as that of Example 2, 15 weight % glucose aqueous solution containing 0.01 weight % aluminum sulfate was introduced from the bottom of this reactor, ethanol fermentation was performed using yeast and the ethanol generated at the reactor outlet was measured and compared.

Table 3 shows the results.

第 3 表

固定化微生物製品	エタノール生成濃度 重量%
従来品	5
本発明品	7

Table 3

Row 1: fixed microorganisms standard product ethanol production concentration weight %
Row 2: conventional product
Row 3: product of present invention

As is clear from the results of Table 3, the ethanol production concentration is smaller in the conventional product than in the product of the present invention. This is due to the fact that in the case of the conventional product of the microorganisms fixed in the form of beads, CO_2 gas is accumulated in a part of the reaction bed by the CO_2 gas bubbles produced during the reaction, disrupting the reaction solution and causing back mixing. In the case of the product of the present invention, such a phenomenon was not observed.

As compared with the conventional fixed microorganisms, the fixed microorganisms of the present invention have the following effects, since the microorganisms containing enzymes are adhere-fixed on the cell wall surface of a honeycomb structured body in a film form with the microorganisms included in polysaccharides:

1. they show high mechanical strengths and minimal activity decreases since the gel-like mixtures are adhere-fixed on cell wall surfaces.
2. as compared with the conventionally fixed microorganisms in the form of beads, pellets etc., the microorganisms fixed in accordance with the present invention show minimal obstruction of the pathway by solid substance as well as minimal pressure loss.
3. the fixed microorganisms of the present invention show minimal disruption of the reaction solution and minimal back mixing since gas generated easily rises and escapes

through the holes perforated, in enzyme reactions that produce gas during the reaction or the microorganism reactions.
4. the fixed microorganisms of the present invention show superior enzyme activities due to large contact surfaces.

Thus, the present invention can be utilized efficiently in various kinds of enzyme reactions, especially, alcohol fermentation that produces gas during the reaction, methane fermentation etc., saccharification reactions of starch with high viscosity substrates etc. or the so-called microorganism reactions.